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(54) Title: MULTIMERIC FORMS OF MEMBERS OF THE STEROID/THYROID SUPERFAMILY OF RECEPTORS

(57) Abstract

In accordance with the present invention, it has been discovered that various members of the steroid/thyroid superfamily of receptors can interact to form multimeric species comprising a complex of more than one receptor. Accordingly, the interaction of a first receptor species with a second receptor species modulates the ability of the first receptor species to trans-activate transcription of genes maintained under hormone expression control in the presence of the cognate ligand for said first receptor.

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MULTIMERIC FORMS OF MEMBERS OF THE STEROID/THYROID SUPERFAMILY OF RECEPTORS

FIELD OF THE INVENTION

The present invention relates to interactions between members of the steroid/thyroid superfamily of receptor proteins, novel combinations of various members of the steroid/thyroid superfamily of receptor proteins, and methods of using such combinations.

BACKGROUND OF THE INVENTION

Transcriptional regulation of development and homeostasis in complex eukaryotes, including humans and other mammals, birds, fish, insects, and the like, is controlled by a wide variety of regulatory substances, including steroid and thyroid hormones. These hormones exert potent effects on development and differentiation of phylogenetically diverse organisms. The effects of hormones are mediated by interaction with specific, high affinity binding proteins referred to as receptors.

A number of receptor proteins are known, each specific for steroid hormones [e.g., estrogens (estrogen receptor), progesterones (progesterone receptor), glucocorticoid (glucocorticoid receptor), androgens (androgen receptor), aldosterones (mineralocorticoid receptor), vitamin D (vitamin D receptor)], retinoids (e.g., retinoic acid receptor) or thyroid hormones (e.g., thyroid hormone receptor. Receptor proteins have been found to be distributed throughout the cell population of complex eukaryotes in a tissue specific fashion.

Molecular cloning studies have made it possible to demonstrate that receptors for steroid, retinoid and thyroid hormones are all structurally related and comprise a superfamily of regulatory proteins. These regulatory

proteins are capable of modulating specific gene expression in response to hormone stimulation by binding directly to cis-acting elements.

An important advance in the characterization of this superfamily of regulatory proteins has been the identification of a growing list of gene products which possess the structural features of hormone receptors.

It is known that steroid or thyroid hormones, protected forms thereof, or metabolites thereof, enter cells and bind to the corresponding specific receptor protein, initiating an allosteric alteration of the protein. As a result of this alteration, the complex of receptor and hormone (or metabolite thereof) is capable of binding with high affinity to certain specific sites on chromatin.

It is also known that many of the primary effects of steroid and thyroid hormones involve increased transcription of a subset of genes in specific cell types.

A number of transcriptional control units which are responsive to members of the steroid/thyroid superfamily of receptors have been identified. These include the mouse mammary tumor virus 5'-long terminal repeat (MTV LTR), responsive to glucocorticoid, aldosterone and androgen hormones; the transcriptional control units for mammalian growth hormone genes, responsive to glucocorticoids, estrogens and thyroid hormones; the transcriptional control units for mammalian prolactin genes and progesterone receptor genes, responsive to estrogens; the transcriptional control units for avian ovalbumin genes, responsive to progesterones; mammalian metallothionein gene transcriptional control units, responsive to glucocorticoids; and mammalian hepatic α_{2u}-globulin gene transcriptional control units, responsive to

androgens, estrogens, thyroid hormones, and glucocorticoids.

A major obstacle to further understanding and more widespread use of the various members of the steroid/thyroid superfamily of hormone receptors has been a lack of awareness of the possible interactions of various members of the steroid/thyroid superfamily of hormone receptors, and an understanding of the implications of such interactions on the ability of members of the steroid/thyroid superfamily of hormone receptors to exert transcriptional regulation of various physiological processes.

BRIEF DESCRIPTION OF THE INVENTION

In accordance with the present invention, we have discovered that two or more members of the steroid/thyroid superfamily of receptors can combine to form multimeric species comprising a complex of more than one receptor. Accordingly, the combination of a first receptor species with a second receptor species is capable of modulating the ability of the first receptor species to trans-activate transcription of genes maintained under expression control in the presence of cognate ligand for said first receptor.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows gel mobility shift assays employing bacterially expressed COUP-TF and RXR, and a ^{32}P-labelled oligonucleotide having a sequence which is recognized by the DNA-binding domains of both COUP-TF and RXR.

Figure 2 summarizes the effect of COUP-TF and EAR-2 on RXR-mediated transactivation studies through an RXR response element.

Figure 3 contains evidence of heterodimer formation between RAR and RXR. Specifically, Figure 3A shows the results of immunoprecipitation reactions between RXR and various other members of the steroid/thyroid superfamily of receptors (including fragments thereof).

Figure 3B shows gel mobility shift assays using in vitro synthesized RAR and/or RXR and a labelled response element (CRBP-II-RXRE).

Figure 3C shows gel mobility shift competition using a labelled response element and an excess of unlabelled competitor response element.

Figure 3D shows gel mobility shift assays using in vitro synthesized RAR and/or RXR and a labelled response element (βRARE).

Figure 3E shows gel mobility shift assays using labelled response element (βRARE) and whole cell extracts prepared from COS cells in which receptor is overexpressed.

Figure 4 provides evidence of heterodimer formation between RXR - TR, and RXR - VDR. Specifically, Figure 4A shows the results of immunoprecipitation reactions between RXR and TR or VDR.

Figure 4B shows gel mobility shift assays using in vitro synthesized RXR, TR, VDR, and GR (as noted) and labelled oligonucleotides encoding various response elements.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the present invention, there are provided multimeric receptor species which belong to the steroid/thyroid superfamily of receptors, comprising at least the dimerization domain of at least two different members of the steroid/thyroid superfamily of receptors.

As employed herein, the term "dimerization domain" of a member of the steroid/thyroid superfamily of receptors refers to that portion of the receptor which is believed to be involved in the formation of multimeric receptor species. This domain typically comprises the carboxy-terminal portion of the receptor, i.e., that portion of a receptor which is 3' with respect to the DNA-binding domain of the receptor.

In accordance with the present invention, there are also provided combination(s) of receptors comprising at least two different members of the steroid/thyroid superfamily of receptors, wherein said receptors are associated in the form of a multimer;

> wherein said combination does not include the binary combination wherein one of said members is selected from RARα, RARβ or RARγ and the other member is selected from TRα or TRβ.

Combinations contemplated by the present invention can broadly be referred to as "multimeric species", which is intended to embrace all of the various oligomeric forms in which members of the steroid/thyroid superfamily of receptors (including fragments thereof comprising the dimerization domains thereof) are capable of associating. Thus, reference to "combinations" of steroid receptors or "multimeric" forms of steroid receptors includes homodimeric combinations of a single receptor (including fragments thereof comprising the dimerization

domains thereof), heterodimeric combinations of two different receptors (including fragments thereof comprising the dimerization domains thereof), homotrimeric combinations of a single receptor (including fragments thereof comprising the dimerization domains thereof), heterotrimeric combinations of two or three different receptors (including fragments thereof comprising the dimerization domains thereof), homotetrameric combinations of a single receptor (including fragments thereof comprising the dimerization domains thereof), heterotetrameric combinations of two or more different receptors (including fragments thereof comprising the dimerization domains thereof), and the like.

As employed herein, the phrase "members of the steroid/thyroid superfamily of receptors" refers to all of the various isoforms of hormone binding proteins that operate as ligand-dependent transcription factors, including members of the steroid/thyroid superfamily of receptors for which specific ligands have not yet been identified (referred to hereinafter as "orphan receptors"). Each such protein has the intrinsic ability to bind to a specific DNA sequence in a target gene. Following binding, the transcriptional activity of the gene is modulated by the presence or absence of the cognate hormone (ligand). The DNA-binding domains of all members of this superfamily of receptors are related, consisting of 66-68 amino acid residues, and possessing about 20 invariant amino acid residues, including nine cysteines. A member of the superfamily can be identified as a protein which contains these diagnostic amino acid residues, which are part of the DNA-binding domain of such known steroid receptors as the human glucocorticoid receptor (amino acids 421-486), the estrogen receptor (amino acids 185-250), the mineralocorticoid receptor (amino acids 603-668), the human retinoic acid receptor (amino acids 88-153), and the like. The highly conserved amino acids of the DNA-binding domain

of members of the superfamily are as follows:

Cys - X - X - Cys - X - X - Asp* - X -
Ala* - X - Gly* - X - Tyr* - X - X -
X - X - Cys - X - X - Cys - Lys* - X -
Phe - Phe - X - Arg* - X - X - X - X -
X - X - X - X - X - (X - X -) Cys - X -
X - X - X - X - (X - X - X -) Cys - X -
X - X - Lys - X - X - Arg - X - X -
Cys - X - X - Cys - Arg* - X - X -
Lys* - Cys - X - X - X - Gly* - Met
(SEQ ID No 1);

wherein X designates non-conserved amino acids within the DNA-binding domain; the amino acid residues denoted with an asterisk are residues that are almost universally conserved, but for which variations have been found in some identified hormone receptors; and the residues enclosed in parenthesis are optional residues (thus, the DNA-binding domain is a minimum of 66 amino acids in length, but can contain several additional residues).

Exemplary members of the steroid/thyroid superfamily of receptors (including the various isoforms thereof) include steroid receptors such as glucocorticoid receptor, mineralocorticoid receptor, progesterone receptor, androgen receptor, vitamin D_3 receptor, and the like; plus retinoid receptors, such as the various isoforms of RAR (e.g., RARα, RARβ, or RARγ), the various isoforms of RXR (e.g., RXRα, RXRβ, or RXRγ), and the like; thyroid receptors, such as TRα, TRβ, and the like; as well as other gene products which, by their structure and properties, are considered to be members of the superfamily, as defined hereinabove, including the various isoforms thereof. Examples of orphan receptors include HNF4 [see, for example, Sladek et al., in Genes & Development 4: 2353-2365 (1990)], the COUP family of receptors [see, for example,

Miyajima et al., in Nucleic Acids Research 16: 11057-11074 (1988), and Wang et al., in Nature 340: 163-166 (1989)], COUP-like receptors and COUP homologs, such as those described by Mlodzik et al., in Cell 60: 211-224 (1990) and Ladias et al., in Science 251: 561-565 (1991), various isoforms of peroxisome proliferator-activated receptors (PPARs; see, for example, Issemann and Green, in Nature 347: 645-650 (1990)), the ultraspiracle receptor [see, for example, Oro et al., in Nature 347: 298-301 (1990)], and the like. Presently preferred members of the superfamily for use in the practice of the present invention are those members which recognize "direct repeat" hormone response elements, as described in detail hereinbelow.

The formation of multimeric species can modulate the ability of the first receptor to trans-activate transcription of genes maintained under expression control in the presence of ligand for said first receptor. The actual effect on activation of transcription (i.e., enhancement or repression of transcription activity) will vary depending on the receptor species which are part of the multimeric species, as well as on the response element with which the multimeric species interacts. Thus, for example, formation of a heterodimer of RXR and RAR inhibits the ability of RXR to trans-activate RXR-mediated processes, while the same heterodimer provides enhanced trans-activation activity with respect to the ability of RAR to trans-activate RAR-mediated processes.

In accordance with another embodiment of the present invention, there is provided a method to modulate, in an expression system, the transcription activation of a gene by a first member of the steroid/thyroid superfamily of receptors, wherein the expression of said gene is maintained under the control of a hormone response element, said method comprising:

exposing said system to at least the

dimerization domain of a second member of the steroid/thyroid superfamily of receptors, in an amount effective to form a multimeric complex with said first member.

Exposure of said system to at least the dimerization domain of a second member of the steroid/thyroid superfamily of receptors is accomplished by directly administering said second member (or dimerization domain thereof) to said system, or by exposing said system to compound(s) and/or condition(s) which induce expression of said second member (or dimerization domain thereof). The resulting multimeric species is effective to modulate transcription activation of said gene by the first member of the steroid/thyroid superfamily of receptors.

As employed herein, the term "modulate" refers to the ability of a given multimeric species to either enhance or repress a receptor's ability fo induce transcription of a target gene, relative to such ability of said receptor in its uncomplexed state. The actual effect of multimerization on a receptor's transcription activity will vary depending on the specific receptor species which are part of the multimeric species, and on the response element with which the multimeric species interacts. Thus, for example, formation of a heterodimer of RXR and TR inhibits the ability of RXR to trans-activate RXR-mediated processes, while the same heterodimer provides enhanced trans-activation activity with respect to the ability of TR to trans-activate TR-mediated processes.

In accordance with one embodiment of the present invention, the first member of the steroid/thyroid superfamily of receptors is an isoform of RXR and the second member is selected from COUP-TF, EAR-2, PPAR, VDR, TR, RAR, or isoforms thereof. Those of skill in the art can readily identify the compound(s) and/or condition(s)

which induce expression of one or more of the second members set forth above.

In accordance with this embodiment, the first member is encoded by a gene expressed in the liver, spleen, kidney, and/or small intestine. The encoded product(s) are involved in lipid metabolism and/or cholesterol homeostasis.

In accordance with another embodiment of the present invention, the first member of the steroid/thyroid superfamily of receptors is an isoform of RAR and said second member is an isoform of RXR. Those of skill in the art can readily identify the compound(s) and/or condition(s) which are capable of inducing expression of one or more isoforms of the second member (RXR) as set forth above.

In accordance with still another embodiment of the present invention, the first member of the steroid/thyroid superfamily of receptors is an isoform of TR and the second member is an isoform of RXR. Those of skill in the art can readily identify the compound(s) and/or condition(s) which are capable of inducing expression of one or more isoform of the second member (RXR) as set forth above.

In accordance with yet another embodiment of the present invention, the first member of the steroid/thyroid superfamily of receptors is VDR and the second member is an isoform of RXR. Those of skill in the art can readily identify the compound(s) and/or condition(s) which are capable of inducing expression of one or more isoform of the second member (RXR) as set forth above.

Hormone response elements contemplated for use in the practice of the present invention include naturally

occurring response elements, or synthetic response elements which are composed of two or more "half sites", wherein each half site comprises the sequence

-RGBNNM-,

wherein

R is selected from A or G;

B is selected from G, C, or T;

each N is independently selected from A, T, C, or G; and

M is selected from A or C;

with the proviso that at least 4 nucleotides of said -RGBNNM- sequence are identical with the nucleotides at corresponding positions of the sequence -AGGTCA-, and

wherein the nucleotide spacing between each of said half-sites falls in the range of 0 up to 15 nucleotides, N.

When one of the half sites varies by 2 nucleotides from the preferred sequence of -AGGTCA-, it is preferred that the other half site of the response element be the same as, or vary from the preferred sequence by no more than 1 nucleotide. It is presently preferred that the 3'-half site (or downstream half site) of a pair of half sites vary from the preferred sequence by at most 1 nucleotide.

Since the half sites are combined in direct repeat fashion (rather than as palindromic constructs), the resulting synthetic response elements are referred to as "DR-x", wherein "DR" refers to the direct repeat nature of the association between the half sites, and "x" indicates the number of spacer nucleotides between each half site.

Exemplary response elements useful in the practice of the present invention are derived from various combinations of half sites having sequences selected from, for example, -AGGTCA-, -GGTTCA-, -GGGTTA-, -GGGTGA-,

-AGGTGA-, -GGGTCA-, and the like.

The nucleotides employed in a non-zero spacer are independently selected from C, T, G, or A.

Exemplary three nucleotide spacers include -AGG-, -ATG-, -ACG-, -CGA-, and the like. Exemplary four nucleotide spacers include -CAGG-, -GGGG-, -TTTC-, and the like. Exemplary five nucleotide spacers include -CCAGG-, -ACAGG-, -CCGAA-, -CTGAC-, -TTGAC-, and the like.

Exemplary response elements contemplated by the present invention include the following DR-3 elements:

5'-AGGTCA-AGG-AGGTCA-3' (SEQ ID No. 2),
5'-GGGTGA-ATG-AGGACA-3' (SEQ ID No. 3),
5'-GGGTGA-ACG-GGGGCA-3' (SEQ ID No. 4),
5'-GGTTCA-CGA-GGTTCA-3' (SEQ ID No. 5),

the following DR-4 elements:

5'-AGGTCA-CAGG-AGGTCA-3' (SEQ ID No. 6),
5'-AGGTGA-CAGG-AGGTCA-3' (SEQ ID No. 7),
5'-AGGTGA-CAGG-AGGACA-3' (SEQ ID No. 8),
5'-GGGTTA-GGGG-AGGACA-3' (SEQ ID No. 9),
5'-GGGTCA-TTTC-AGGTCC-3' (SEQ ID No. 10),

the following DR-5 elements:

5'-AGGTCA-CCAGG-AGGTCA-3' (SEQ ID No. 11),
5'-AGGTGA-ACAGG-AGGTCA-3' (SEQ ID No. 12),
5'-GGTTCA-CCGAA-AGTTCA-3' (SEQ ID No. 13),
5'-GGTTCA-CCGAA-AGTTCA-3' (SEQ ID No. 14),
5'-AGGTCA-CTGAC-AGGGCA-3' (SEQ ID No. 15),
5'-GGGTCA-TTCAG-AGTTCA-3' (SEQ ID No. 16),
5'-AAGCTTAAG-GGTTCA-CCGAA-AGTTCA-CTCAGCTT-3' (SEQ ID No. 17),
5'-AAGCTTAAG-GGTTCA-CCGAA-AGTTCA-CTCGCATAGCTT-3' (SEQ ID No. 18),
5'-AAGCTTAAG-GGTTCA-CCGAA-AGTTCA-CTCGCATATATTAGCTT-3' (SEQ ID No. 19), and the like.

Presently preferred response elements contemplated for use in the practice of the present invention include:

5'-AGGTCA-AGG-AGGTCA-3' (SEQ ID No. 2),
5'-AGGTCA-CAGG-AGGTCA-3' (SEQ ID No. 6),
5'-AGGTGA-CAGG-AGGTCA-3' (SEQ ID No. 7),
5'-AGGTCA-CCAGG-AGGTCA-3' (SEQ ID No. 11),
5'-AGGTGA-ACAGG-AGGTCA-3' (SEQ ID No. 12),

and the like. These are especially preferred because they represent synthetic sequences which have not been observed in nature, and thus are applicable to a wide variety of reporter systems (i.e., the use of these response elements will not be limited due to any species preference based on the source of the sequence).

The invention will now be described in greater detail by reference to the following non-limiting examples.

EXAMPLES

Plasmids

Receptor expression plasmids used in the cotransfection assays are described by Mangelsdorf et al. [see Cell Vol. 66:555-561 (1991)]; and Umesono et al. [see Cell Vol. 65:1-20 (1991)].

RS-COUP-TF expression plasmid was constructed by inserting an Asp718-BamHI fragment containing the EAR-3 (i.e., COUP) coding region [Miyajima et al., Nucl. Acids Res. Vol. 16:11057-11074 (1988)] into Asp718-BamHI-cut pRS expression vector.

To construct the RS-EAR-2 expression plasmid, an Eco47III-BglII fragment containing the EAR-2 coding region (Miyajima et al., supra) was blunted with Klenow and inserted into Asp718-BamHI-cut pRS, which had also been

end-filled with Klenow.

All of the recombinant reporter constructs used contain either one or two copies of the indicated oligonucleotides inserted at the unique HindIII site upstream of the basal reporter construct ΔSV-CAT (Umesono et al., supra). Identity and orientation of the inserted oligonucleotides was confirmed by sequencing.

Cotransfection Assays

CV-1, HeLa, and F9 teratocarcinoma cell culture, transfections, and CAT assays were performed as previously described (Mangelsdorf et al., supra: Umesono et al., supra). In cotransfection experiments including expression plasmids RS-COUP-TF and RS-EAR-2 (see Figure 2), cell extracts were normalized to total amount of protein for use in CAT assays, as these expression constructs were shown to severely repress expression of β-galactosidase expression vectors.

Bacterial Expression of RXR and COUP-TF

hRXRα was expressed in bacteria as a fusion protein with glutathione-S-transferase using the pGEX-2T expression vector [Smith and Johnson, Gene Vol. 67:31-40 (1988)]. Purification of the fusion protein and cleavage of the glutathione-S-transferase protein from RXR with thrombin were performed as described by Mangelsdorf et al., supra.

For expression of COUP-TF in bacteria, a 1.8 kb NcoI-BamHI fragment containing the entire coding region of EAR-3 (Miyajima et al., supra) was inserted into the PET-8C expression vector [Studier et al., Methods in Enzymology 185: 60-89 (1990). BL21(DE3)plysS cells [Studier et al., supra] containing the PET-8C-COUP-TF expression construct

were induced for 3 hours with 0.6 mM isopropylthiogalactoside (IPTG) and the cells subsequently lysed in lysis buffer [50 mM Tris (pH 8.0), 250 mM KCl, 1 mM DTT, 1 mM PMSF, 1% Triton X-100] by freeze-thawing.

Lysates were clarified by centrifugation for 1 hour at 45,000 rpm in a Ti60 rotor (Beckman). Crude bacterial lysates containing COUP-TF were diluted in lysis buffer lacking KCl to a final concentration of 100 mM KCl and loaded on a heparin-agarose column. The column was washed with Buffer A [20 mM Tris (pH 8.0), 20% glycerol, 1 mM DTT, 1 mM PMSF], and COUP-TF subsequently eluted with Buffer A containing 800 mM KCl.

The eluted protein was dialyzed to 100 mM KCl, loaded on a MonoQ column (Pharmacia), and protein eluted with a linear salt gradient (100 mM-800 mM) in Buffer A. Fractions containing COUP-TF binding activity (eluting at 300-350 mM KCl) were pooled and aliquoted for use in gel mobility shift assays. Western blot analysis done using COUP-TF-specific antiserum confirmed that the partially-purified COUP-TF migrated upon SDS-PAGE as an ~45 kD protein.

DNA-Binding Assays

Gel mobility shift assays (20 µl) contained 10 mM Tris (pH 8.0), 40 mM KCl, 0.1% NP-40, 6% glycerol, 1 µg of poly(dI-dC), and the specific receptor species indicated in the figure legends. After 10 minutes incubation on ice, 1 ng of ^{32}P-labeled oligonucleotide was added and the incubations were continued for an additional 10 minutes. DNA-protein complexes were resolved on 4% polyacrylamide gels in 0.5 x TBE (1 x TBE = 90 mM Tris, 90 mM boric acid, 2 mM EDTA). Gels were dried and subjected to autoradiography at -70°C. The following oligonucleotides and their complements were ^{32}P-labeled and used as probes:

DR-0: AGCTTC-AGGTCA-AGGTCA-GAGAGCT (SEQ ID No. 20);

DR-1: AGCTTC-AGGTCA-G-AGGTCA-GAGAGCT (SEQ ID No. 21);

DR-2: AGCTTC-AGGTCA-GG-AGGTCA-GAGCT (SEQ ID No. 22);

DR-3: AGCTTC-AGGTCA-AGG-AGGTCA-GAGAGCT (SEQ ID No. 23);

DR-4: AGCTTC-AGGTCA-CAGG-AGGTCA-GAGAGCT (SEQ ID No. 24);

DR-5: AGCTTC-AGGTCA-CCAGG-AGGTCA-GAGAGCT (SEQ ID No. 25);

βRARE: AGCTTAAG-GGTTCA-CCGAA-AGTTCA-CTCGCATAGCTGCT (SEQ ID No. 26);

COUP-TF RE:

AGCTTG-GTGTCA-A-AGGTCA-AACTTAGCT (SEQ ID No. 27);

CRBPII-RXRE:

AG-CTGTCA-C-AGGTCA-C-AGGTCA-C-AGGTCA-C-AGTTCA-AGCT (SEQ ID No. 28).

RXR Antiserum

A peptide corresponding to amino acids 214-229 of hRXRα was synthesized according to the technique of Rivier et al. [Science Vol. 224:889-891 (1984)]. A glycine and tyrosine were added to the carboxy terminus for coupling to human α-globulins using bisdiazotized benzadine as described by Vaughan et al., in Methods in Enzymology Vol. 168:588-617 (1989). For initial injection, Freund's complete adjuvant was mixed with an equal volume of physiological saline containing 1 mg conjugate/ml. For boosters, Freund's incomplete adjuvant was mixed with an equal volume of physiological saline containing 0.5 mg conjugate/ml. For each immunization, a rabbit received a total of 1 ml emulsion in multiple intradermal sites. Animals were injected every three weeks and bled through an ear vein seven days after each boost. Serum was collected and evaluated for receptor antibodies on the basis of Western blot analysis of hRXRα transfected COS cell extracts. The antisera used herein was collected after the sixth boost.

EXAMPLE I

COUP-TF and RXR form a heterodimer in vitro

Bacterial-expressed COUP-TF and RXR-glutathione-S-transferase fusion protein (RXR-GST) were mixed and the resulting complexes analyzed by gel mobility shift assays using ^{32}P-labeled DR-1 oligonucleotide (i.e., SEQ ID No. 21) as probe. The larger RXR fusion protein was used in order to maximize the migratory differences observed between the COUP-TF and RXR complexes. RXR-GST behaved identically to the nonfusion protein in terms of binding specificity with all the response elements tested, including exhibiting a marked preference for DR-1 relative to the other DRs.

Gel mobility shift assays were performed using ^{32}P-labeled DR-1 oligonucleotide (SEQ ID No. 21) in the presence of partially-purified COUP-TF (500 ng) and increasing amounts of partially-purified RXR (1X - 50ng) as indicated in Figure 1. Either 0.3 µl or 1 µl of RXR-specific antiserum was included in the assays (shown in lanes 11 and 12, respectively). The positions of the RXR-specific and COUP-TF-specific complexes are indicated in Figure 1 by a plain line ("-"). The position of the COUP-TF-RXR heterodimeric complex is indicated in the Figure by an arrow, and the position of supershifted complexes is indicated in the Figure by an arrowhead. The free probe was run off the gel and is not shown.

As shown in Figure 1 (lane 2), low amounts of RXR-GST bound only weakly to DR-1, although at higher concentrations a homodimeric complex was seen (lane 8). However, addition of increasing amounts of RXR-GST to a constant amount of COUP-TF resulted in the appearance of a complex with mobility intermediate to those formed by COUP-TF and RXR-GST alone, with the concomitant loss of the COUP-TF-specific complex (lanes 3, 6 and 9). Addition of purified GST alone did not affect the mobility of the COUP-

TF complex. Formation of COUP-TF-RXR heterodimers was clearly favored relative to the formation of either homodimeric complex under the conditions employed.

Addition of RXR-specific antiserum to an assay containing both COUP-TF and RXR-GST resulted in the "supershifting" of the COUP-TF-RXR complex (lane 11). The RXR-specific antiserum did not cross-react with bacterially-expressed COUP-TF. Increasing the amount of antiserum added to the gel mobility shift assay ultimately resulted in the disruption of the COUP-TF-RXR interaction and reappearance of the COUP-TF-specific complex (lane 12). The release of COUP-TF from this complex is a likely consequence of higher amounts of the antibody stabilizing RXR homodimers.

Similar supershift data, indicating the formation of a COUP-TF-RXR heterodimeric complex, were also obtained using radiolabeled ovalbumin COUP-TF RE as probe. These results, taken together, provide compelling evidence that COUP-TF and RXR can form a highly stable heterodimeric complex in vitro.

EXAMPLE II

COUP-TF represses RXR-mediated transactivation through an RXR-RE

The observation that RXR can stimulate transcription through a COUP-TF recognition element suggests that COUP-TF might reciprocally activate through a CRBPII site. The in vitro binding data presented above strongly supports this proposal. However, in cotransfection analyses, it is not possible to obtain a significant COUP-TF-mediated activation of expression from reporter plasmids COUP RE2-ΔSV-CAT or CRBPII-ΔSV-CAT when tested in either F9, CV-1, or HeLa cells (Figure 2A, lanes 9 and 10). A closely related receptor, referred to as

EAR-2 (Miyajima et al., supra), also fails to activate transcription through the CRBPII reporter (Figure 2A, lanes 11 and 12). Because COUP-TF and EAR-2 are orphan receptors, it is possible that efficient transactivation through the COUP-TF and CRBPII response elements will require addition of exogenous ligand.

As an alternative approach, it was investigated whether COUP-TF could alter RXR-mediated induction from the CRBPII-RXRE. Accordingly, the CRBPII-CAT reporter, containing the intact promoter region of the CRBPII gene, was cotransfected into F9 cells with either RXR expression plasmid alone, or in combination with expression plasmids for either COUP-TF or EAR-2. F9 cells were cotransfected in duplicate with 3 μg the reporter pCRBPII-CAT and 1 μg of RS-hRXRα plus 0.5 μg of either the control RS-LUC (lanes 1 and 2), RS-hRARα (lanes 3 and 4), RS-COUP-TF (lanes 5 and 6), or RS-EAR-2 (lanes 7 and 8). Transfection of each 10 cm plate also included 5 μg of RAS-ß-galactosidase and 5.5 μg of pUC19 as carrier. Cotransfections performed with the reporter pCRBPII-CAT and either 0.5 μg RS-COUP-TF (lanes 9 and 10) or 0.5 μg of RS-EAR-2 (lanes 11 and 12) in the absence of RS-hRXRα are also shown in Figure 2. Cells were treated with either ethanol (-) or 10 μM RA (+) for 30 hours and the cell extracts subsequently assayed for CAT activity. One set of the duplicate CAT assays is shown in the Figure.

As expected, addition of retinoic acid (RA) to cells cotransfected with CRBPII-CAT reporter and RXR expression plasmid resulted in a dramatic (approximately 90-fold) induction of CAT activity (Figure 2A, compare lanes 1 and 2). RXR-mediated activation through the CRBPII promoter could, however, be blunted by cotransfection of RAR expression plasmid (lanes 3 and 4). Remarkably, inclusion of expression plasmids encoding either COUP-TF or EAR-2 in the cotransfection assay completely eliminated

RXR-mediated transactivation through the CRBPII promoter (lanes 5-8). Thus, both COUP-TF and EAR-2 can function as potent repressors of RXR-mediated transactivation through the intact CRBPII promoter.

To demonstrate that this repression was mediated by the CRBPII element, a parallel experiment utilizing the CRPBII-ΔSV-CAT reporter was performed in CV-1 cells. CV-1 cells were cotransfected in duplicate with the reporter CRBPII-ΔSV-CAT and RS-hRXRα (1μg) in the presence of 0.5 μg RS-LUC [as a control; designated in the figure as (C)], or 0.2 and 0.5 μg of RS-COUP-TF or RS-EAR-2. Cells were treated with either ethanol (-) or 10 μM RA (+) and the cell extracts subsequently assayed for CAT activity. CAT activity is shown in Figure 2B as percent maximal conversion where the RA-inducible activity obtained from CRBPII-ΔSV-CAT in the presence of RS-hRXRα alone is arbitrarily set at 100%.

Similar results were obtained, with both COUP-TF and EAR-2 functioning as potent inhibitors of RXR-mediated activation (Figure 2B). As shown in Figure 2C, the presence of either COUP-TF or EAR-2 failed to significantly reduce overall levels of RAR-mediated transactivation through the βRARE, although a slight (2- to 3-fold) increase in CAT activity in the absence of RA was reproducibly seen. CV-1 cells were cotransfected in duplicate with the reporter βRARE-ΔSV-CAT (Umesono et al., supra) and RS-RARα (1 μg) plus 0.5 μg of either RS-LUC [as a control; designated in the figure as (C)], RS-COUP-TF or RS-EAR-2. Cells were treated with either ethanol (-) or 10 μM RA (+) and the cell extracts subsequently assayed for CAT activity. CAT activity is shown in Figure 2C as percent conversion where the RA-inducible activity obtained from βRARE-ΔSV-CAT in the presence of RS-RARα alone is arbitrarily set at 100%.

These results indicate that COUP-TF/EAR-2-mediated suppression of reporter activity is specific for RXR and its response element.

EXAMPLE III

Evidence for RXR-TR and RXR-VDR Heterodimer Formation

Immunoprecipitation experiments were performed using bacterially-expressed RXR and ^{35}S-methionine-labeled RAR synthesized *in vitro*. RAR, LBD, or GR RNA was prepared and subsequently translated in rabbit reticulocyte lysates as directed by the supplier (Promega). RXR was expressed in bacteria as a fusion with glutathione-S-transferase using the pGEX-2T expression vector (Pharmacia) as described by Mangelsdorf et al., *supra*. Immunoprecipitation reactions (20 µl) included 5 µl of [^{35}S]methionine-labeled receptor protein and 150 ng of either purified GST-RXR or GST alone in 20 mM Tris, pH 8.0. Proteins were incubated 20 minutes on ice prior to the addition of 5 µl of polyclonal RXR antiserum. Antigen-antibody complexes were collected by the addition of Protein A-Sepharose (Pharmacia) and the immunocomplexes washed three times with 400 µl RIPA buffer [10 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate]. Immunoprecipitated complexes were resolved by SDS polyacrylamide gel electrophoresis on 10% gels which were then fixed in 30% methanol, 10% acetic acid, dried, and subjected to autoradiography. Gel retardation assays (20 µl) contained 10 mM Tris (pH 8.0), 40 mM KCl, 0.1% NP-40, 6% glycerol, 0.2 mM EDTA, 0.1 mM DTT, 0.2 µg of poly(dI-dC) and 2.5 µl of *in vitro* synthesized RAR and RXR proteins. When either RAR or RXR was omitted, the reaction was supplemented with the same volume of unprogrammed reticulocyte lysate. After a 10 minute incubation on ice, 1 ng of ^{32}P-labeled oligonucleotide was added and the incubation continued for an additional 10 minutes. DNA-

protein complexes were resolved on a 4% polyacrylamide gel in 0.5X TBE (1X TBE = 90 mM Tris, 90 mM boric acid, 2 mM EDTA). Gels were dried and subjected to autoradiography at -70°. Gel mobility shift assays performed using Cos cell-expressed receptors were performed as described by Umesono et al., supra using whole cell extracts prepared from Cos cells transfected with either RS-hRARa, RS-hRXRa, or both expression plasmids.

As shown in Figure 3A, preincubation of RXR and RAR followed by precipitation with anti-RXR antiserum resulted in the efficient co-precipitation of radiolabeled RAR (Figure 3A, lane 2). In contrast, no RAR was detected when RXR was omitted from the reaction (Figure 3A, lane 1).

Similar experiments in which RAR was replaced with radiolabeled GR failed to reveal RXR-GR interactions, demonstrating the specificity of the RAR-RXR interaction under these conditions (see Figure 3A, lanes 5 and 6). Consistent with transfection data indicating the importance of the carboxy-terminus of RAR in mediating RAR-RXR interactions, a truncated RAR protein, consisting of only the C-terminal region of RAR, was also efficiently co-precipitated with RXR (Figure 3A, lanes 3 and 4). Thus, RAR and RXR form a highly stable heterodimer in solution; the carboxy-terminus of RAR, containing the ligand binding and dimerization domains, is sufficient for this interaction.

The stability of the RAR-RXR heterodimer in solution suggested that the two proteins might also interact and display novel properties when associated with DNA. To test this possibility, gel mobility shift experiments were first performed using in vitro synthesized RAR and RXR and a radiolabeled oligonucleotide encoding the CRBPII-RXRE (i.e., SEQ ID No. 28). As shown in Figure 3B, RAR synthesized in vitro bound with very low affinity to

CRBPII-RXRE (lane 3). However, the affinity of binding of RAR to CRBPII-RXRE was dramatically enhanced by the addition of in vitro synthesized RXR (Figure 3B, lane 4). In vitro synthesized RXR alone had no detectable binding activity (Figure 3B, lane 2). Inclusion of polyclonal antisera prepared against either RAR or RXR in the reaction mixture resulted in the disruption of the protein-DNA complex and appearance of novel complexes with reduced mobility (Figure 3B, lanes 5 and 6), indicating that both RAR and RXR were present in the complex. Thus, the RAR-RXR heterodimer is capable of interacting with high affinity with the CRBPII-RXRE.

The results of the transfection analyses presented above indicate that, under the conditions employed, the RAR-RXR heterodimer is transcriptionally inactive on the CRBPII-RXRE.

The specificity of the RAR-RXR interaction with DNA was next examined using unlabeled oligonucleotides as competitor. Oligonucleotides containing the CRBPII-RXRE (SEQ ID No. 28) competed efficiently for RAR-RXR heterodimer binding at a 10-fold molar excess (Figure 3C, lane 2), whereas oligonucleotides containing an unrelated glucocorticoid response element (GRE; Schüle et al., Cell 62:1217-1226 (1990)) failed to compete when used at a 40-fold molar excess relative to the radiolabeled CRBPII-RXRE (Figure 3C, lane 7). Interestingly, oligonucleotides containing the RARE of the RARß promoter (ßRARE; SEQ ID No. 26) also competed efficiently for RAR-RXR binding to the CRBPII (Figure 3C, lanes 4 and 5).

To further investigate this interaction of the RAR-RXR heterodimer with the ßRARE (i.e., SEQ ID No. 26), oligonucleotides containing the ßRARE were labeled and used as probe in a gel mobility shift assay. As in the case of the CRBPII-RXRE, both in vitro synthesized RAR and RXR were

required for high affinity DNA-protein interactions with the βRARE (Figure 3D, lanes 2-4).

Similar results indicating a requirement for the presence of both RAR and RXR for formation of a high affinity DNA-protein complex on the βRARE were obtained using whole-cell extracts prepared from Cos cells which had been transfected with either RAR alone, RXR alone, or both RAR and RXR (Figure 3E). Taken together, these results demonstrate that RXR dramatically enhances the binding affinity of RAR to a strong retinoic acid response element, and that the RAR-RXR complex is likely to be present in vivo.

Similarly, in immunoprecipitation experiments, in vitro synthesized thyroid receptor-beta (TRβ) and vitamin D receptor (VDR) were found to co-precipitate with bacterially-expressed RXR (Figure 4A, lanes 1-6). The interactions of these receptors with RXR were also manifest at the level of DNA binding: in vitro synthesized RXR was shown to dramatically enhance TRβ and VDR binding to the MLV-LTR TRE (Umesono et al., supra) and osteopontin VDRE (Umesono et al., supra), respectively (Figure 4B, lanes 1-8).

Taken together, these data strongly suggest a central role for members of the steroid/thyroid superfamily of receptors, such as RXR, in modulating the hormonal responses conferred via the RAR, TR, and VDR.

While the invention has been described in detail with reference to certain preferred embodiments thereof, it will be understood that modifications and variations are within the spirit and scope of that which is described and claimed.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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Kliewer, Steven A.
Umesono, Kazuhiko

(ii) TITLE OF INVENTION: MULTIMERIC FORMS OF MEMBERS OF THE STEROID/THYROID SUPERFAMILY OF RECEPTORS

(iii) NUMBER OF SEQUENCES: 28

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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

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(A) APPLICATION NUMBER: US 07/803,163
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Cys Xaa Xaa Cys Xaa Xaa Asp Xaa Ala Xaa Gly Xaa Tyr Xaa Xaa Xaa
1 5 10 15

Xaa Cys Xaa Xaa Cys Lys Xaa Phe Phe Xaa Arg Xaa Xaa Xaa Xaa Xaa
20 25 30

Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys
35 40 45

Xaa Xaa Xaa Lys Xaa Xaa Arg Xaa Xaa Cys Xaa Xaa Cys Arg Xaa Xaa
50 55 60

Lys Cys Xaa Xaa Xaa Gly Met
65 70

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

AGGTCAAGGA GGTCA 15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GGGTGAATGA GGACA 15

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GGGTGAACGG GGGCA 15

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGTTCACGAG GTTCA 15

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGGTCACAGG AGGTCA 16

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AGGTGACAGG AGGTCA 16

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGGTGACAGG AGGACA 16

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGGTTAGGGG AGGACA 16

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GGGTCATTTC AGGTCC 16

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGGTCACCAG GAGGTCA 17

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AGGTGAACAG GAGGTCA 17

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GGTTCACCGA AAGTTCA 17

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGTTCACCGA AAGTTCA 17

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGGTCACTGA CAGGGCA 17

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGGTCATTCA GAGTTCA 17

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AAGCTTAAGG GTTCACCGAA AGTTCACTCA GCTT 34

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AAGCTTAAGG GTTCACCGAA AGTTCACTCG CATAGCTT 38

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AAGCTTAAGG GTTCACCGAA AGTTCACTCG CATATATTAG CTT 43

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AGCTTCAGGT CAAGGTCAGA GAGCT 25

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGCTTCAGGT CAGAGGTCAG AGAGCT 26

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

AGCTTCAGGT CAGGAGGTCA GAGCT 25

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AGCTTCAGGT CAAGGAGGTC AGAGAGCT 28

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

AGCTTCAGGT CACAGGAGGT CAGAGAGCT 29

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

AGCTTCAGGT CACCAGGAGG TCAGAGAGCT 30

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AGCTTAAGGG TTCACCGAAA GTTCACTCGC ATAGCTGCT 39

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

AGCTTGGTGT CAAAGGTCAA ACTTAGCT 28

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

AGCTGTCACA GGTCACAGGT CACAGGTCAC AGTTCAAGCT 40

That which is claimed is:

1. A combination of receptors comprising at least two different members of the steroid/thyroid superfamily of receptors, wherein said receptors are associated in the form of a multimer; and

wherein said combination does not include the binary combination wherein one of said members is selected from RARα, RARß or RARγ and the other member is selected from TRα or TRß.

2. A combination of receptors according to claim 1 wherein said combination is in the form of a heterodimer.

3. A combination of receptors according to claim 1 wherein said combination is in the form of a heterotrimer.

4. A combination of receptors according to claim 1 wherein said combination is in the form of a heterotetramer.

5. A combination of receptors according to claim 1 wherein one of said members is selected from RXRα, RXRß or RXRγ.

6. A combination of receptors according to claim 5 wherein another of said members is selected from COUP-TF, PPAR or EAR-2.

7. A combination of receptors according to claim 5 wherein another of said members is VDR.

8. A combination of receptors according to claim 5 wherein another of said members is TRα or TRß.

9. A combination of receptors according to claim 5 wherein one of said members is RARα, RARß or RARγ.

10. A combination of receptors according to claim 1 wherein one of said members is RXRα and another of said members is RXRß or RXRγ.

11. A combination of receptors according to claim 1 wherein one of said members is RARα and another of said members is RARß or RARγ.

12. A multimer comprising at least the dimerization domain of at least two different members of the steroid/thyroid superfamily of receptors.

13. A method to modulate, in an expression system, the transcription activation of a gene by a first member of the steroid/thyroid superfamily of receptors, wherein the expression of said gene is maintained under the control of a hormone response element, said method comprising:

exposing said system to compound(s) and/or condition(s) which induce expression of at least the dimerization domain of a second member of the steroid/thyroid superfamily of receptors, in an amount effective to form a multimeric complex with said first member.

14. A method according to claim 13 wherein said hormone response element has the sequence:

$$5'\text{-NNNNNN-}(N_x\text{-NNNNNN})_y\text{-}3',$$

wherein

each N is independently selected from A, T, C, or G; with the proviso that at least 3 nucleotides of each -NNNNNN- group of nucleotides are identical with the nucleotides at comparable positions of the sequence -AGGTCA-,

x is zero or a whole number in the range of 1 up to 15, and

y is a whole number of at least 1.

15. A method according to claim 13 wherein said first member is RXR and said second member is selected from COUP-TF, PPAR, EAR-2, VDR, TR, or RAR.

16. A method according to claim 15 wherein said gene is expressed in at least the liver and small intestine.

17. A method according to claim 16 wherein said gene encodes product(s) involved in lipid metabolism.

18. A method according to claim 16 wherein said gene encodes product(s) involved in cholesterol homeostasis.

19. A method according to claim 13 wherein said first member is RAR and said second member is RXR.

20. A method according to claim 13 wherein said first member is TR and said second member is RXR.

21. A method according to claim 13 wherein said first member is VDR and said second member is RXR.

FIG. 1

Lane	1	2	3	4	5	6	7	8	9	10	11	12
COUP-TF	+	−	+	+	−	+	+	−	+	+	+	+
RXR	−	1X	1X	−	2X	2X	−	4X	4X	+	+	+

FIG. 2A

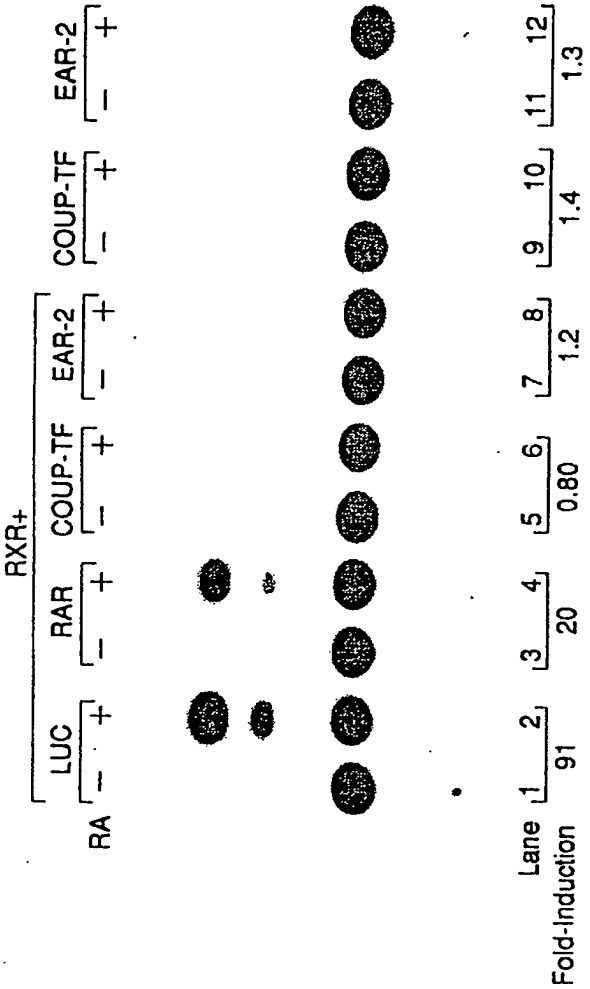

RXR+
LUC
RAR
COUP-TF
EAR-2
COUP-TF
EAR-2
RA
Lane
Fold-Induction
91
20
0.80
1.2
1.4
1.3

FIG. 2B

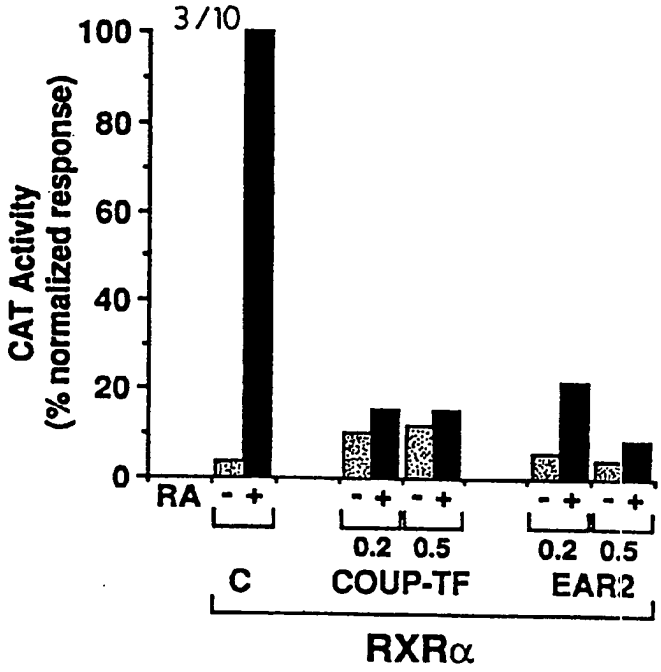
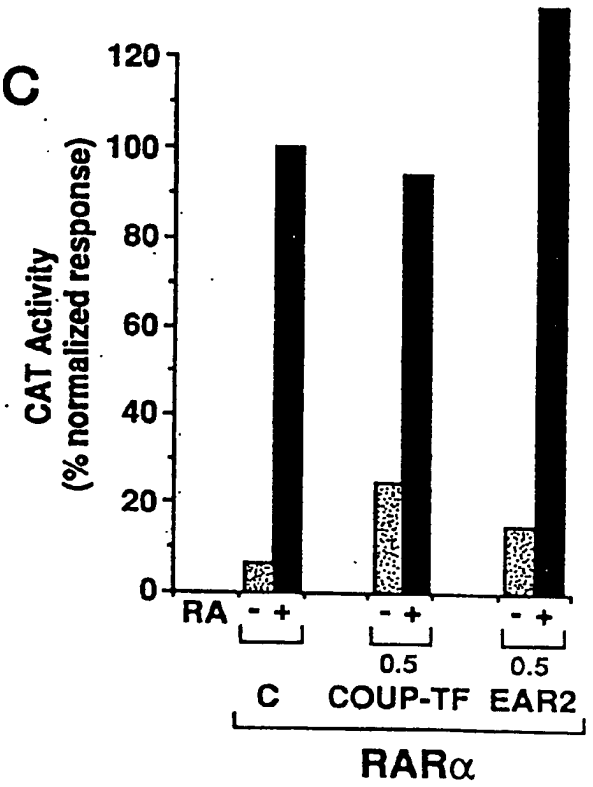

3/10
CAT Activity
(% normalized response)
100
80
60
40
20
0
RA
- +
- + - +
0.2 0.5
- + - +
0.2 0.5
C
COUP-TF
EAR2
RXRα

FIG. 2C


CAT Activity
(% normalized response)
120
100
80
60
40
20
0
RA
- +
- +
0.5
- +
0.5
C
COUP-TF
EAR2
RARα

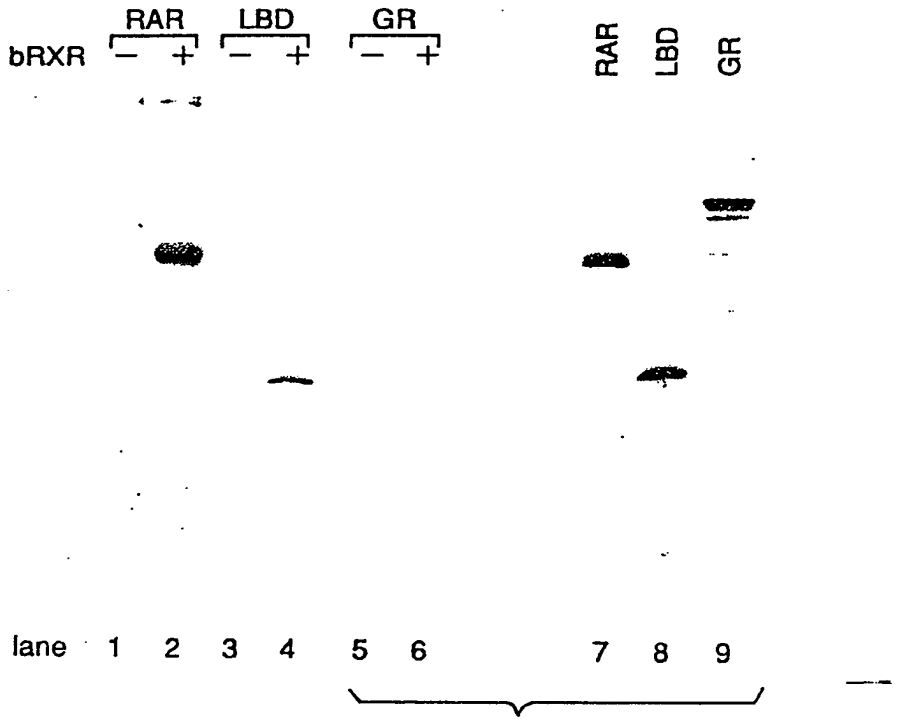
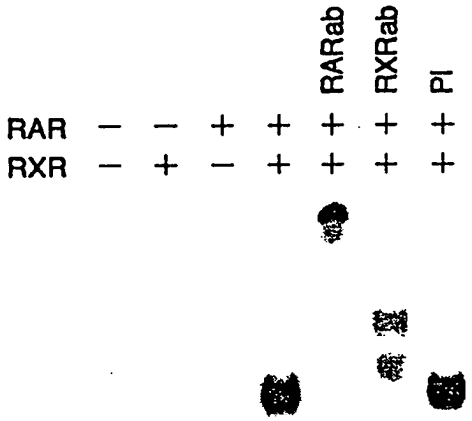


FIG. 3A



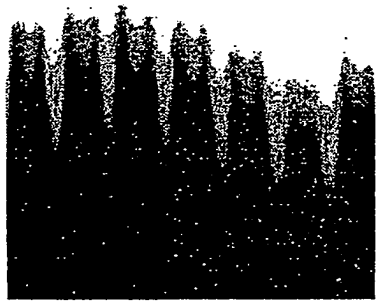


FIG. 3B

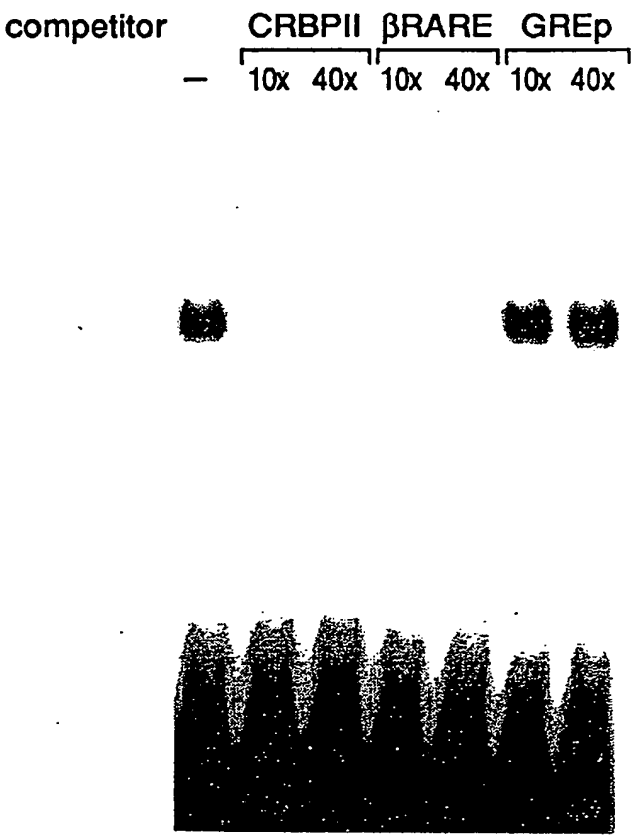

competitor
CRBPII βRARE GREp
–
10x 40x 10x 40x 10x 40x
lane
1 2 3 4 5 6 7

FIG. 3C

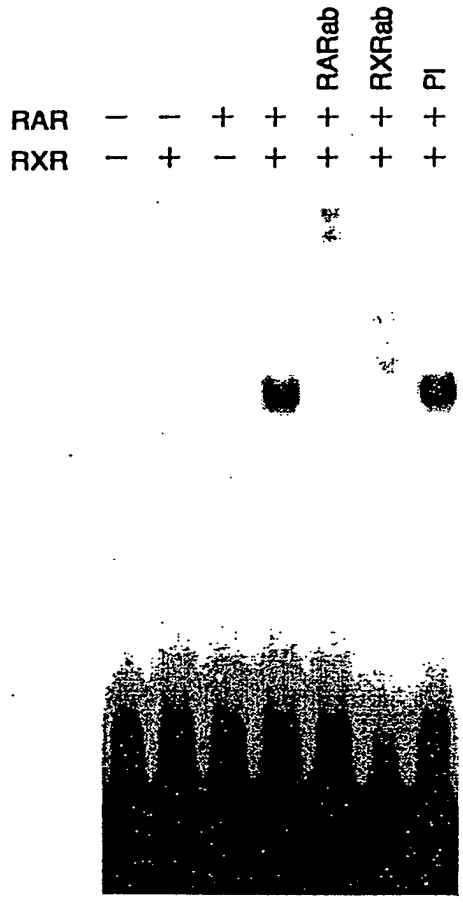


FIG. 3D

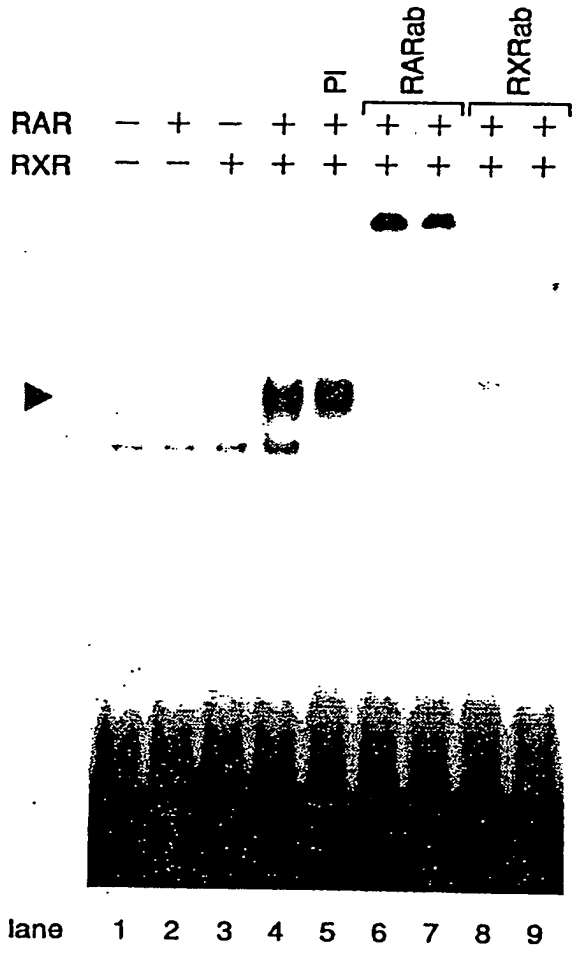


FIG. 3E

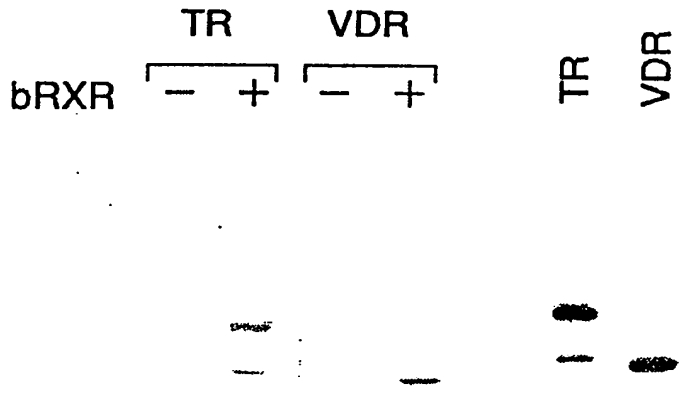
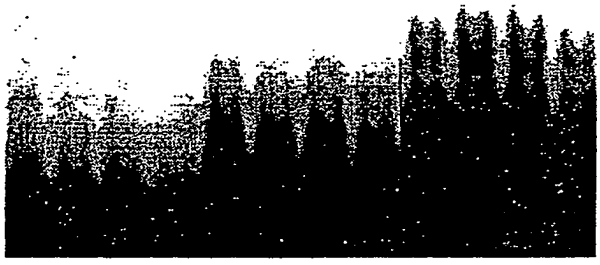


FIG. 4A

GR								−	−	+	+
VDR					−	−	+	+				
TR	−	−	+	+								
RXR	−	+	−	+	−	+	−	+	−	+	−	+



lane 1 2 3 4 5 6 7 8 9 10 11 12

FIG. 4B

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/10508

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/12; C12N15/62; C07K13/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; C07K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category[*]	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	CELL vol. 59, 17 November 1989, CAMBRIDGE, NA US pages 697 - 708 Glass, C.K. et al.; 'Positive and negative regulation of gene transcription by a retinoic acid-thyroid hormone receptor heterodimer."' see the whole document ---	1-13, 19-21
X	CELL vol. 63, 16 November 1990, CAMBRIDGE, NA US pages 729 - 783 Glass, K.C. et al.; 'Multiple cell type-specific proteins differentially regulate target sequence recognition by the alpha retnoic acid receptor.' see the whole document --- -/--	1-13, 19-21 — —

[*] Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23 MARCH 1993	29. 03. 93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	S.A. NAUCHE

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category°	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	MOLECULAR AND CELLULAR BIOLOGY vol. 11, no. 10, October 1991, WASHINGTON US pages 5005 - 5015 Lazar MA;Berrodin TJ;Harding HP; 'Differential DNA binding by monomeric, homodimeric, and potentially heteromeric forms of the thyroid hormone receptor.' see the whole document	1-13, 19-21
X	FASEB JOURNAL vol. 5, no. 14, 14 November 1991, BETHESDA, MD US pages 2924 - 2933 De Luca LM; 'Retinoids and their receptors in differentiation, embryogenesis, and neoplasia.' see the whole document	1-14, 19-21
X	JOURNAL OF BIOLOGICAL CHEMISTRY. (MICROFILMS) vol. 266, no. 34, 5 December 1991, BALTIMORE, MD US pages 23296 - 23305 Sone T;Kerner S;Pike JW; 'Vitamin D receptor interaction with specific DNA. Association as a 1,25-dihydroxyvitamin D3-modulated heterodimer.' see the whole document	1-13, 19-21

INTERNATIONAL SEARCH REPORT

Int. ational application No.
PCT/US 92/ 10508

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 13-15, 19-21 partially(as far as they are related to animal/human body) and 16-18 completely are directed to a method of treatment of the human/animal body (PCT-Rule 39.1(iv)) the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.